IN SITU HYBRIDIZATION

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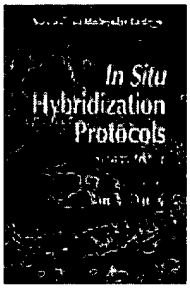
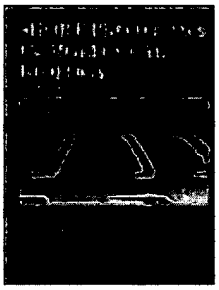
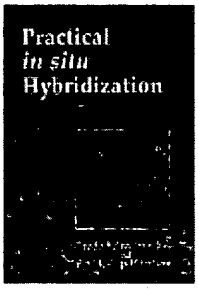
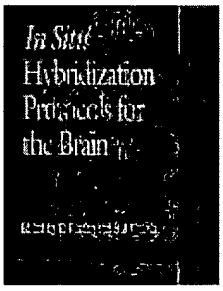








In situ hybridization - the issues

Updated 22 November 2004

Introduction

In situ hybridization, as the name suggests, is a method of localizing and detecting specific mRNA sequences in morphologically preserved tissues sections or cell preparations by hybridizing the complimentary strand of a nucleotide probe to the sequence of interest.

Normal hybridization requires the isolation of DNA or RNA, separating it on a gel, blotting it onto nitrocellulose and probing it with a complimentary sequence.

The basic principles for *in situ* hybridization are the same, except one is utilizing the probe to detect specific nucleotide sequences within cells and tissues. The sensitivity of the technique is such that threshold levels of detection are in the region of 10-20 copies of mRNA per cell.

In situ hybridization presents a unique set of problems as the sequence to be detected will be at a lower concentration, be masked because of associated protein, or protected within a cell or cellular structure. Therefore, in order to probe the tissue or cells of interest one has to increase the permeability of the cell and the visibility of the nucleotide sequence to the probe without destroying the structural integrity of the cell or tissue.

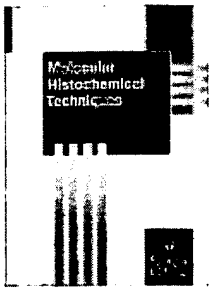
You also have to consider the type of probe to use, and how best to label it, to give the best level of resolution with the highest level of stringency. Some of these choices are dependent on the type of questions being asked, facilities available, and how one intends to assess the outcome, ie qualitatively or quantitatively.

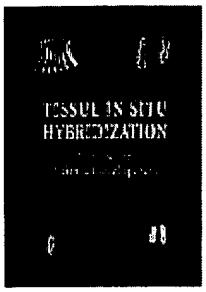
There are almost as many methods for carrying out *in situ* hybridization as there are tissues that have been probed. So more important than having a recipe is to have an understanding of the different stages in the process and their purpose.

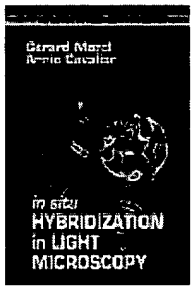
Preparation of Material.

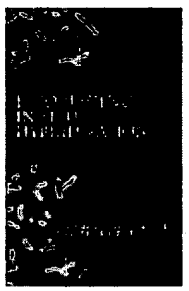
The most common tissue sections used with *in situ* hybridization are

a) Frozen sections. Fresh tissue is snap frozen (rapidly put into a -80 freezer) and









then when frozen embedded in a special support medium for thin cryosectioning. The sections are lightly and rapidly fixed in 4% paraformaldehyde just prior to processing for hybridization.

b) Paraffin embedded sections. Sections are fixed in formalin as one would normally fix tissues for histology and then embedded in wax (paraffin sections) before being sectioned or

c) Cells in suspension. Cells can be cytospun onto glass slides and fixed with methanol

Choice of Probe

Probes are complimentary sequences of nucleotide bases to the specific mRNA sequence of interest. These probes can be as small as 20-40 base pairs or be up to 1000 bp. Although ultimately the question you ask and the type of sequence you are trying to detect is the overriding factor, one needs to optimize, as much as possible the conditions one uses. The strength of the bonds between the probe and the target plays an important role. The strength decreases in the order RNA-RNA to DNA-RNA. This stability is in turn influenced by the various hybridization conditions such as concentration of formamide, salt concentration, hybridization temperature, and pH

Probe types

There are essentially four types of probe that can be used in performing in situ hybridization. We will briefly detail all of your options before explaining why in most cases in situ hybridization performed using correctly designed and purified oligonucleotide probes will represent the easiest, fastest, least labor intensive and most inexpensive method.

1. Oligonucleotide probes

These are produced synthetically by an automated chemical synthesis. The method utilizes readily available deoxynucleotides which are economical, but of course requires that you know the specific nucleotide sequence you wish to prepare. Designing the sequence of the probe is one of the more critical decisions required when using oligonucleotide probes and is just not a matter of picking any region within the coding region of the target gene to bind to but requires careful design taking into account a number of issues (read below). These probes have the advantages of being resistant to RNases and are small, generally around 40-50 base-pairs. This is ideal for *in situ* hybridization because their small size allows for easy penetration into the cells or tissue of interest. In addition, because they are synthetically designed, it is possible to make a series of probes that have the same GC content; Since G/C base pairs bond more strongly than A/U base pairs, differences in GC content would require different hybridization conditions, so with oligonucleotides protocols can be standardized for many different probes irrespective of the target genes being measured. Another advantage of the oligonucleotide probes is that they are single stranded therefore excluding the possibility of renaturation.

2. Single stranded DNA probes.

These have similar advantages to the oligonucleotide probes except they are much larger, probably in the 200-500 bp size range. They can be produced by reverse transcription of RNA or by amplified primer extension of a PCR-generated fragment in the presence of a single antisense primer. That is, once you have amplified the sequence of interest, a subsequent round of PCR is carried out using the first PCR product as template, but only using the anti-sense primers, thus producing single stranded DNA. This is therefore their disadvantage. They require time to prepare, expensive reagents are used during their preparation and a good repertoire of molecular skills are required for their use.

3. Double stranded DNA probes

These can be produced by the inclusion of the sequence of interest in a bacteria which is replicated, lysed and the DNA extracted, purified and the sequence of interest is excised with restriction enzymes. On the other hand, if the sequence is known then by designing appropriate primers one can produce the relevant sequence very rapidly by PCR, potentially obtaining a very clean sample. The advantage of the bacterial preparation is that it is possible to obtain large quantities of the probe sequence in question. Because the probe is double stranded, it means that denaturation or melting has to be carried out prior to hybridization in order for one strand to hybridize with the mRNA of interest. These probes are generally less sensitive because of the tendency of the DNA strands to rehybridize to each other and are not as widely used today.

4. RNA probes (cRNA probes or riboprobes)

RNA probes have the advantage that RNA-RNA hybrids are very thermostable and are resistant to digestion by RNases. This allows the possibility of post-hybridization digestion with RNase to remove non-hybridized RNA and therefore reduces the possibility of background staining.

There are two methods of preparing RNA probes:

1. Complimentary RNA's are prepared by an RNA polymerase-catalyzed transcription of mRNA in the 3' to 5' prime direction.
2. Alternatively, in vitro transcription of linearized plasmid DNA with RNA polymerase can be used to produce the RNA probes. Here plasmid vectors containing polymerase from bacteriophages T3, T7 or SP6 are used

These probes however can be very difficult to work with as they are very sensitive to RNases (ubiquitous RNA degrading enzymes) and so scrupulous sterile technique should be observed or these probes can easily be destroyed. So saying this, RNA probes are still probably the most widely used probes with in situ hybridization. Nevertheless below we want to demonstrate to you why the correct oligonucleotide probe is the preferred probe choice for your in situ hybridization experiment.

Benefits of using oligonucleotide probes

While in situ hybridization is undoubtedly a very powerful technique, for the average laboratory it is expensive to undertake, is time consuming, requires detailed molecular biological knowledge of subcloning, in-vitro transcription and bacterial expression. The probes most often used (RNA or cDNA) are not generally available commercially and are often obtained on an ad hoc basis, laboriously prepared on a case by case basis by the investigator and often once purchased require time-consuming and expensive preparation before use. In addition, as mentioned above RNA probes are sensitive to ubiquitous RNAses necessitating the baking of labware and extreme attention to detail to prevent any contamination of solutions. This includes the addition of expensive RNase inhibitors (i.e. DEPC, diethylpyrocarbonate) to all critical solutions. As anybody that has performed in situ hybridization with RNA probes (riboprobes) knows they can be very frustrating to work with.

Furthermore, and this is an important point, if somewhat counter-intuitive, it can be shown that because of their long size (100s of base-pairs as opposed to oligonucleotide probes that are generally 40-50bps long) RNA and cDNA probes are often less specific than shorter oligonucleotide probes. Why? Well because they can often cross-recognize (bind non-selectively to) close members of the same gene family even under stringent hybridizing conditions. Oligonucleotides on the other hand (for example those custom designed by GeneDetect) can be targeted to those DNA sequence within a gene family that are most variable. For example we use various bioinformatic techniques and oligonucleotide optimization design software to design our proprietary GeneDetect™ gene probes (i.e. those not available in the public domain) specifically to distinguish between members of the same gene family.

Longer RNA probes also show poor tissue penetration and while chemical shortening (hydrolysis) enhances tissue penetration it also increases the likelihood of non-selective binding to other non-targeted gene sequences.

What is required therefore to make in situ hybridization an easier technique to work with for the general lab worker is a better all round method for measuring tissue mRNA expression. The technique must be faster, cheaper, more robust and have greater gene specificity. We believe that the correct use of oligonucleotide gene probes and *in situ* hybridization represents this solution.

Oligonucleotide gene probes have multiple advantages over RNA or cDNA probes when used for in situ hybridization. We can list them here for you. Stability, Availability, Faster and less expensive to use, Easier to work with, More specific, Better tissue penetration, Better reproducibility and a wide range of labeling methods that do not interfere with target detection.

1. Stability. Oligonucleotide probes are very stable. They are not degraded by RNases. Once tissue is fixed, solutions do not need to be RNase free. Simple autoclaving of solutions will do. Oligonucleotide probes in our experience do not degrade when stored lyophilized at -20C. In fact we have not yet seen probe degradation when our probes have been stored in solution at -20C after 3 years. Our experience tells us that the main reason for probe degradation is the addition of bacterial contamination to the probe solution by the use of non-autoclaved pipette tips. If you follow general laboratory sterile technique your probe should last for many years at -20C.

2. Availability. They are available for purchase. We intend to make oligonucleotide probe packs to newly cloned important genes available as soon as these gene sequence are entered into GenBank. If your probe is not available you can request that we design a probe for you. These sequences can be emailed to you. Furthermore we will synthesize and purify the probe for optimal use with in situ hybridization and ship it to you within a week from first contact. Our probes are >95% full length meaning that you do not get a random mixture of half synthesized probes from us that could lead to increased background staining or cross hybridization to non-targeted genes. The purification of probes after chemical synthesis is also important. Our probes are all cartridge purified. When you receive an oligonucleotide probe there is no "growing-up" of the probe in bacteria or further purification or subcloning required. Simply use the probe as directed in our protocol sheets.

3. Faster and less expensive to use. The technique of in situ hybridization becomes far cheaper and faster when oligonucleotide probes are used. A full in situ hybridization experiment can be done in 2 days depending on the detection step. Approximately the time it takes to perform immunocytochemistry. The costs to the laboratory are dramatically reduced when oligonucleotide probes are used instead of RNA probes because of the high cost of synthesis, purification and labeling of RNA probes and because of the extra chemicals used in the RNA in situ hybridization procedure.

4. Easier to work with. No Molecular Biological knowledge is required to perform in situ hybridization with oligonucleotide probes. If you don't believe us, see our protocol for performing in situ hybridization on fresh frozen tissue sections.

5. More specific. As mentioned, unlike RNA probes, oligonucleotide probes can be designed to selectively recognize members of closely related gene families since they can be designed against regions of high variability within the gene family. This is not the case with RNA probes which are often directed against the entire coding region or against some highly conserved sequences. Specificity of binding of oligonucleotide probes to the target gene in tissue sections with in situ hybridization can be further strengthened with the use and comparison of binding of two probes complementary to different regions within the given target gene in any given experiment.

6. Better tissue penetration. Oligonucleotide probes penetrate tissue far more easily than RNA probes increasing the sensitivity of signal detection into the region of 10-20 copies of mRNA or DNA per cell.

7. Better reproducibility. In situ hybridization with oligonucleotide probes is highly reproducible in every tissue and with every probe. It is possible to make a series of probes that have the same GC content; Since G/C base pairs bond more strongly than A/U base pairs, differences in GC content (often found when comparing two cDNA or RNA probes) would require different hybridization conditions to allow direct comparison of the hybridization signals. Furthermore with oligonucleotide probes the control of the relevant parameters (probe concentration, specific activity and melting temperature, Tm) can allow the determination of quantitative characteristics of the binding such as apparent Kd and Bmax.

8. Labeling methods do not interfere with target detection. Methods used in labeling RNA and cDNA probes for detection in in situ hybridization result in strongly labeled probes but may not always be the best for in situ hybridization because the labeled nucleotides may interfere with the hybridization reaction. This is not an issue for oligonucleotide probes (see below).

Labeling your oligonucleotide

Options

To "see" where the probe has hybridized (bound) within your tissue section or within your cells and thus to determine where your target gene is being expressed you must attach to the probe an easily detectible substance or "label" before hybridization.

Classically oligonucleotide probes have been either 5' or 3' end-labeled or 3' tailed with modified nucleotides that have a "label" attached that can be detected after the probe has hybridized to its target. With end-labeling a single modified ddNTP (that incorporates the label) is added to either the 5' or the 3' end of the molecule enzymatically or during probe synthesis. 3' tailing involves addition of a tail (on average 5-50 nucleotides long of modified dNTPs depending on the method used) using the enzyme terminal transferase (TdT).

We have recently developed a new non-enzymatic proprietary labeling technology, GreenGene™ that allows for covalent 3' labeling of oligonucleotides subsequent to probe synthesis. Using this technology an optimized number of labels (either Biotin, FITC, rhodamine or DIG) separated by proprietary linker molecules can be added to the 3' end of an oligonucleotide probe in one chemical step. Click here to learn more about the GreenGene™ labeling technology and our new GreenStar™ labeled probe selection. GreenStar™ labeled probes have been optimized for use with in situ hybridization.

Traditionally oligonucleotide probes have been radiolabeled. Radiolabeled probes are still the choice for many workers. ^{35}Sulphur (^{35}S) is the most commonly used radioisotope because its high activity is necessary to detect transcripts present in low amounts. Radiolabeled probes are visualized by exposure of the tissue section or cells (to which the labeled oligonucleotide has be hybridized) against photographic film which is then developed (See Figure).

Figure. Localization of ActRIIA activin receptor mRNA in rat brain using a ^{35}S-dATP 3'-tailed GeneDetect™ gene probe. After hybridization the tissue was exposed to photographic film for 7 days.

Instead of using photographic film to detect the probe within the tissue section, the slide containing the section of interest may also be dipped into a photographic emulsion which is allowed to dry. The slide is stored in the dark at -80C to allow the slide emulsion to become exposed. After the incubation period, the slides are then developed in the same way as normal photographic film. Thus you view the section or cells of interest through the developed photographic emulsion and the black silver grains indicate the sites of the labeled transcripts. This is particularly useful for investigating gene expression on a cell by cell level. If immunocytochemistry is performed on the tissue before in situ hybridization, it is possible to examine gene expression in phenotypically defined cell populations at single cell resolution.

When using radiolabeling, waste disposal and containment measures must be given thought and it must be remembered that the useful shelf life of your labeled probe is inherently dependent on the half life of the radionucleotide. For ^{35}S this amounts to an experimental life time of about 80 days for your labeled probe before it would be wise to again relabel the probe with newly purchased radionucleotide. So saying this we still find using ^{35}S labeled oligonucleotide probes very quick, easy and very sensitive.

On the other hand several non-radioactive labels used successfully with in situ hybridization include Biotin, digoxin and digoxigenin (DIG), alkaline phosphatase and the fluorescent labels, fluorescein (FITC), Texas Red and rhodamine. Since these non-radioactive labels have no inherent "decay" kinetics, once a probe has been labeled it can either be used immediately or be divided into aliquots, lyophilized and stored at -20C for later use for as long as 1-2 years with careful storage.

Labeling the probe

GreenStar™ labeled probes.

Our new GreenStar™ labeled GeneDetect™ oligonucleotide probes have been optimized for use with in situ hybridization. Purchase of a GreenStar™ Biotin, FITC, rhodamine or DIG-labeled probe removes the need to label the probe yourself and standard techniques for label detection can still be used (see below).

5' end labeling

In addition we are able to prepare 5' end labeled probes (addition of one Biotin, rhodamine, FITC modified nucleotide to the 5' end) during our synthesis cycle if required. 5' end labeled probes have been shown to work quite well with in situ hybridization when target transcript levels are reasonably high and/or an amplification step is incorporated into the protocol. For example with 5' Biotin labeled probes signal

amplification can be achieved by complexes of ExtrAvidin®-Horseradish Peroxidase (HRP) and a biotinylated anti-Avidin antibody as found in the "in situ Hybridization Detection Kit for Biotin-labeled probes" supplied by Sigma-Aldrich.

If you decide to label the probe yourself, these are some of the labeling methods available to you.

3' tailing.

Both radiolabels and non-radioactive labels are "attached" to the single stranded oligonucleotide probe by using the enzyme terminal transferase to add a tail of labeled dioxy nucleotide(s) (dNTPs, for example ^{35}S-dATP, DIG-dUTP, Biotin-dUTP or FITC-dUTP) to the 3' end (3' tailing) of the oligonucleotide. This simple procedure adds a 3'-OH tail to the end of the oligonucleotide probe which ranges in length from 10-100 nucleotides (average of about 50). Many commercial kits are available that allow you to 3' tail your probe. 3' tailed probes work quite well with in situ hybridization. Some of the companies that supply oligonucleotide 3' tailing kits include Sigma, Pierce and Roche Molecular Biochemicals (DIG tailing kits).

3' end labeling

It is also possible to end label the 3' end of the oligonucleotide probe if modified di-deoxy nucleotides (ddNTPs) are used as the substrate for TdT, for example by using DIG-ddUTP instead of DIG-dUTP with a slightly modified protocol. In this case TdT will add a single modified and labeled nucleotide to the 3' end of the oligonucleotide facilitating later detection.

Incorporation of label

In contrast to adding the labeled nucleotides to either end of the oligonucleotide probe it is also possible to have labels incorporated into the oligonucleotide when it is being synthesized, for example by adding biotin- or FITC-labeled dATP in place of non-labeled dATP during synthesis so that a label or "tag" appears every time that the ATP nucleotide appears in the probe sequence. However with this method there is the strong possibility especially with short oligonucleotide probes of causing significant disruption of the hybridization of the probe to the target sequence in the tissue.

For these reasons we recommend 3' tailing with TdT as the preferred method for labeling oligonucleotide probes for use with in situ hybridization if you choose to label the probe yourself.

Detection.

As mentioned, radiolabeled probes are detectable using either photographic film or photographic emulsion.

The fluorescent labels described above are detectible "directly" by using a fluorescent microscope or plate reader to examine the tissue or cells on which the labeled oligonucleotide probe has hybridized to. The use of fluorescent labels with in situ hybridization has come to be known as FISH (fluorescent in situ hybridization) and one advantage of these fluorescent labels is that two or more different probes can be visualized at one time. Additionally FITC labeled probes can be detected using anti-FITC antibodies available from many scientific supply houses.

In contrast both Biotin and DIG labeled oligonucleotide probes generally require an intermediate step(s) before detection of the probe can occur and they are thus detected "indirectly" much like in a typical immunocytochemistry protocol where it would be unusual to have the label on the primary antibody, rather a secondary labeled antibody is used to detect the primary antibody in the tissue section. Specific anti-DIG antibodies can be used to detect the presence of a DIG-labeled probe. Digoxigenin (DIG) is a steroid isolated from the digitalis plant and as the blossoms and

leaves are the only known source of digoxigenin, the anti-DIG antibodies are not likely to bind to other biological material. The digoxigenin is linked by a spacer arm containing 11 carbon atoms to the C-5 position of the uridine nucleotide. The advantage of using a DIG labeled probe is that it can be detected with antibodies conjugated to a number of different labels such as alkaline phosphatase, which results in a blue precipitate when the enzyme is incubated in the presence of the substrate NBT/BCIP (Tetrazoliun salt/ 5-bromo- 4-chloro- 3 idolyl-phosphate) or becomes a fluorescent label when incubated with HNPP (2hydroxy-3naphthoic acid-2'phenylanilide phosphate). The level of sensitivity of these labeled probes is purported to be 0.1 pg on a Southern blot but would be about 10 fold less sensitive for in situ hybridization. The Anti-DIG antibodies can be conjugated to other labels that require no development, such as FITC, Texas Red or Rhodamine. In some protocols anti-DIG antibodies are unlabelled and a secondary conjugated anti-anti body is used to visualize the probe. Being closely related in structure to digoxigenin, GreenStar digoxin hyperlabeled probes can be detected using the same methods and kits designed for digoxigenin detection.

Biotin is the other common compound used in the labeling of oligonucleotide probes. Linked to ATP (other nucleotides have also been biotinylated) it can be detected with antibodies but more often a 65 kd glygoprotein Avidin from egg white or Streptavidin from the fungi *Streptomyces avidinii* is used, as they have a high binding capacity to biotin and can be conjugated to a similar range of visual and fluorescent labels.

In general, of the two "indirect" labels it is thought that the DIG label is more sensitive than the Biotin label and that the DIG label allows comparable sensitivity to ^{35}S radiolabeled probes (see above).

Hybridization Issues

Lets imagine we have our tissue section on a slide. We already know the gene whose mRNA expression we want to measure, we have our oligonucleotide probe and we have decided on using one of the labeling methods previously discussed to label our probe or have purchased a GreenStar™ hyperlabeled probe. Apart from the necessary in situ hybridization controls (next section) what other issues need to be discussed before we go ahead with our experiment?

Permeablization.

Well firstly it is important that the probe reaches the target, that is the mRNA of the target gene located in your sample. With a tissue section this may not be much of a problem but if you have whole cells or even whole organisms then there are cell membranes which have to be crossed. The act of fixation results in cross-linking of proteins, which once again may present an obstacle to good infiltration of the probe, and finally mRNA sequences are often surrounded by proteins which may mask the target sequence. Therefore the first of the remaining issues to be discussed is sample permeabilization.

There are a number of different elements in permeabilization procedures, some protocols contain most of them, others only a few, but it is important to know why you are treating your sample with particular solutions in order to assess if your particular tissue or cells require it. Three common reagents used to permeabilize tissue are HCl, detergents (Triton or SDS) and Proteinase K.

HCl. Some protocols call for incubation in 0.2M HCl for 20- 30 min. Although the precise action of the acid is not known, it is thought that extraction of proteins and hydrolysis of the target sequence may help decrease the level of background staining.

Detergent treatment, usually with Triton X-100 or SDS, is frequently used to permeabilize the membranes by extracting the lipids. This is not usually required in tissue that has been embedded in wax, but for intact cells or cryostat sections these may be more critical steps.

Proteinase K is an endopeptidase which is non-specific and attacks all peptide bonds, is active over wide pH range and not easily inactivated. It is used to remove protein that surrounds the target sequence. Optimal concentration have to be determined but a normal starting concentration is 1 μg/ml. Incubation has to be carefully monitored because if the digestion proceeds to far you could end up destroying most of the tissue or cell integrity.

Pretreatment/Prehybridization step(s).

Pretreatment/Prehybridization is generally carried out to reduce background staining. Many of the non-radioactive oligonucleotide probe detection methods utilize enzymes such as peroxidases or alkaline phosphatases to visualize the label. Therefore one has to make certain that any endogenous tissue enzymes which could result in giving a very high background are neutralized. This can be achieved with peroxidases by treating the tissues with 1% H_2O_2 in methanol for 30 minutes. For Alkaline phosphatases, the drug levamisole may be added to the substrate solution. In general, however, this is considered to be unnecessary since residual alkaline phosphatase activity is usually lost during hybridization.

Another commonly observed pre treatment when using RNA probes is acetylation with acetic anhydride (0.25%) in triethanolamine. This treatment is thought to be important for decreasing background but it also appears to inactivate RNases and may help in producing a strong signal. Luckily RNases are not an issue when oligonucleotide probes are used. Fixation of tissue effectively "secures the target RNA" i.e. the mRNA within the tissue from RNAse digestion and oligonucleotide probes are naturally resistant to RNases. This is why solutions do not need to be RNase free when using oligonucleotide probes with in situ hybridization.

Prehybridization involves incubating the tissue/section with a solution that is composed of all the elements of the hybridization solution, minus the probe. Not all protocols use a prehybridization step.

Hybridization

The composition of the hybridization solution is critical in controlling the efficiency of the hybridization process. Hybridization depends on the ability of the oligonucleotide to anneal to a complementary mRNA strand just below its melting point (Tm). The value of the Tm is the temperature at which half of the oligonucleotide is present in a single stranded form.

The factors that influence the hybridization of the oligonucleotide probe to the target mRNA are:

- Temperature
- pH
- monovalent cation concentration
- presence of organic solvents

The following is a typical hybridization solution with a hybridization temperature of around 37C and an overnight incubation period.

- Dextran sulphate. This is added because it becomes strongly hydrated and thus reduces the amount of hydrating water for dissolving the nucleotides and therefore effectively increases the probe concentration in solution resulting in higher hybridization rates.
- Formamide and DTT (dithiothreitol). These are organic solvents which reduce the thermal stability of the bonds allowing hybridization to be carried out at a lower temperature.
- SSC (NaCl + Sodium citrate). Monovalent cations interact mainly with the phosphate groups of the nucleic acids decreasing the electrostatic interactions

between the two strands.
- EDTA. This is a chelator and removes free divalent cations from the hybridization solution, because they strongly stabilize duplex DNA.

Other components are added to decrease the chance of nonspecific binding of the oligonucleotide probe and include:

- ssDNA
- tRNA acts as a carrier RNA
- polyA
- Denhardts solution

Washes

Following hybridization the material is washed to remove unbound probe or probe which has loosely bound to imperfectly matched sequences. Washing should be carried out at or close to the stringency condition at which the hybridization takes place with a final low stringency wash.

Controls

Of course the most important part of any experimental procedure is the inclusion of controls. However often with in situ hybridization experiments controls not used properly, if at all.

In carrying out an in situ hybridization experiment one has to be confident that the hybridization reaction is specific and that the probe is in fact binding selectively to the target mRNA sequence and not to other components of the cell or other closely related mRNA sequences. In addition if no staining is observed with the probe does this mean that there really is no expression of that mRNA in the tissue or does it mean that there may be a problem with tissue preparation or the tissue itself?

If the correct controls are included in the experiment we can, with high certainty, answer these questions. Note that the polyd(T) probe is included with all orders and that the nonsense probe and pan-species actin probe are contained within our Control Probes product, Cat # 5000-OP. Both sense and antisense probes are sent when you order a probe from us in amounts that allow for 10X competition studies to be performed as mentioned below. RNase enzyme should be purchased from a trusted supplier.

Controls for tissue mRNA quality and the efficacy of your protocol.

If the quality of your tissue is poor and/or your RNA is degraded it will be very hard to get good results with in situ hybridization. There are however a number of controls you can add to your experiment to verify the status of your tissue and mRNA within the tissue. If you are using fresh tissue and these controls are negative, then this suggests a problem with your technique or protocol.

Poly(dT) probe.

The poly(dT) probe we supply will detect total mRNA poly A tails. If a very weak signal is obtained using this probe then it is likely your tissue RNA is degraded. The chance of detecting a specific mRNA in this tissue is therefore unlikely.

Probes against house keeping sequences.

Some genes are always expressed constituently such as Actin or beta-tubulin. We offer probes to detect these mRNAs. A low signal once again suggests tissue RNA degradation.

Positive control.

Perform in situ hybridization using the correct oligonucleotide probe on a fresh, positive control tissue known to have the sequence of interest (not always possible). If you detect no signal then this suggests the problem exists within your technique or protocol.

Specificity controls.

Determine that your probe is only binding to RNA.

When probing for mRNAs one can determine that the binding is specific to RNA by digesting the tissue with RNases prior to hybridization with the oligonucleotide probe. The absence of binding after RNase treatment indicates that binding was indeed to RNA within the tissue. Download the protocol.

Specific versus non-specific binding.

The first control involves hybridization of the tissue with both labeled sense and antisense probes in parallel. The antisense probe in theory detects both the target mRNA and any non-specific targets it can bind to due to the chemical properties of the probe (but not due to the probe sequence). The sense control probe gives a measure of non-specific probe binding only due to the chemical properties of the probe. In essence if your sense probe detects nothing, then you can be sure that any signal detected by your antisense probe is due to sequence-specific binding to mRNA and not due to binding to other targets within the cell.

Competition studies with labeled and excess unlabeled probes can also help distinguish between specific versus non-specific binding. This is because by definition specific binding is saturable (i.e. there are finite target mRNA molecules to which the probe can bind) while non-specific binding is not (there are infinite non-specific targets). Therefore excess unlabeled probe can displace (by competition for binding sites) the specific binding of the labeled probe (i.e. to the target mRNA) but not non-specific binding of the labeled probe. We recommend prehybridizing tissue with 10X molar amount of 1. unlabeled correct sequence oligonucleotide probe and 2. unlabelled nonsense probe before hybridizing with labeled probe. The nonsense probe should preferably have a similar CG content, a similar length and have no homology to the sequence of interest. It is important to note however that competition studies do not verify the identity of the mRNA to which the labeled probe is binding since both the labeled and unlabeled probes have the same sequence. Download the protocol.

Determine that your probe is binding to the correct target sequence.

The best way to ensure that your probe is binding to the correct target sequence is by choosing a correct probe sequence from the start and having high stringency hybridization and wash conditions in your experiment. If you purchase your probe from us we ensure both of these conditions are met by designing your probe ourselves or by offering you a probe that has previously been characterized and validated by publication in the scientific literature. All sequences within our product range are checked through GenBank. This gives a printout of genes that the probe will possibly bind to. We always enclose this search with each probe sent to our customers. Any "public domain" probes entered into our database that we have found might possibly be less than selective we will "flag" and give you, the customer, the option of using the "public domain" probe, or an alternative probe designed by us.

In summary we recommend that the following controls are performed in parallel with your in situ experiments.

1. poly(dT) probe hybridized to sections. What is the quality of the mRNA in your tissue sample?

2. RNase treatment of sections before labeled antisense probe hybridized. Is probe binding to mRNA?

3. Hybridize in parallel labeled sense and labeled antisense probes. Is the probe binding to the tissue in a sequence-specific fashion?

4. Hybridize labeled antisense probe in presence of a) 10X unlabeled antisense probe and separately in presence of b) 10X unlabeled nonsense probe. Can sequence-specific binding be displaced?

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